



Received on 23 December, 2015; received in revised form, 04 February, 2016; accepted, 16 February, 2016; published 01 June, 2016

PICOGRAM LEVEL QUANTIFICATION OF VARENICLINE IN HUMAN PLASMA SAMPLES BY LC-ESI-MS/MS

Praveen Kumar Dasari* and Srinivasa Rao Darapaneedi

K.C. Reddy Institute of Pharmaceutical Sciences, Guntur-522348, Andhra Pradesh, India

Key words:

LC-ESI-MS/MS;
Varenicline; Human plasma

Correspondence to Author:

Praveen Kumar Dasari.

Assistant Professor,
K. C. Reddy Institute of
Pharmaceutical Sciences,
Jangamguntlapalem, Medikonduru,
Guntur – 522348, Andhra Pradesh,
India

Email: praveenbiotech007@gmail.com


ABSTRACT: The objective of this research was to develop and validate a simple, sensitive and specific Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) quantification of Varenicline in human plasma using Varenicline -D4 as Internal Standard (IS). The analyte was separated on a Zorbax SB-C18, 4.6 x 75 mm, 3.5 μ m, 80 Å column with an isocratic mobile phase of 5mM ammonium formate: acetonitrile (10:90 v/v) at a flow rate of 0.8 mL/min. The protonated ions were formed by a turbo ionspray in a positive mode was used to detect analyte and internal standard (IS). The analytes were monitored by electrospray ionization in positive ion multiple reaction monitoring (MRM) mode was used to detect the Varenicline at m/z 212.1/169.0. This method is validated over a linear concentration range of 50.0–10000.0 pg/mL with a correlation coefficient (r) of ≥ 0.9997 . Both drug and internal standards were stable in plasma samples. This method demonstrated intra and inter-day precision within 1.2–4.5 and 3.5–7.4 and accuracy within 91.70–105.5% and 103.9–110.6%.

INTRODUCTION: Varenicline tartrate (VT) (7,8,9,10-tetrahydro-6,10-methano-6H-pyrazino [2,3-h] [3] benzazepine) was recently introduced as a novel efficacious smoking cessation aid that acts as an $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChRs) partial agonist, centrally acting as a highly selective partial agonist for the nicotinic acetylcholine receptor¹⁻². Varenicline has mixed agonistic-antagonistic properties, thus it has the therapeutic benefit of relieving the symptoms of nicotine withdrawal and cigarette craving during abstinence while blocking the reinforcing effect of nicotine in those who lapse³.

Varenicline tartrate (Champix® and Chantix®; Pfizer) has been approved by the Food and Drug Administration (FDA) as an aid to smoking cessation⁴.

Maximum plasma concentrations of varenicline tartrate occur typically within 3 to 4 h after oral administration. Mean \pm standard deviation (SD) C_{max} was 9.22 ± 2.05 ng/ml at the recommended dose. The approved regime of VT is 1mg for 12 weeks, starting with a 1-week titration period⁵⁻⁶.

The quality of pharmaceutical product of VT, in terms of purity and stability of the active substance and/or finished product is vital for the effective and safest delivery of its therapeutic values to the smokers. A detailed understanding of correlations of drug levels with drug action is an important aspect of the routine use of drug. The accurate quantification of agents in biological matrices such as blood, serum, urine and tissue samples is the

QUICK RESPONSE CODE 	DOI: 10.13040/IJPSR.0975-8232.7(6).2510-17
	Article can be accessed online on: www.ijpsr.com
DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7(6).2510-17	

cornerstone of therapeutic drug monitoring. Therefore, detailed specific, reproducible and accurate method for the quantification of VT is necessary⁷⁻⁸. Additionally, examining the matrix effects represents an important issue in liquid chromatography tandem mass spectrometric (LC-MS/MS), particularly when dealing with biological matrices such as biological fluids. These phenomena can be reduced by an efficient sample preparation and an adequate chromatographic separation with the elution of the analytes outside the matrix effect time window generally observed at the beginning of the chromatogram⁹⁻¹⁰.

However in quantitative analysis, these conditions might be insufficient to reduce interferences, and other approaches should be combined to compensate residual matrix effects; the use of multiple reaction monitoring (MRM) mode can be one of these approaches two LC-MS/MS methods have been published for the quantification of varenicline in human plasma, the first one was done by which has then been applied for the study of varenicline pharmacokinetics. The second method was developed for determination of nicotine, cotinine, trans-3'-hydroxycotinine and varenicline, which was performed as a procedure for a clinical study on smoking cessation to confirm abstinence from smoking and detection of overdose¹¹. In dosage form, five methods were reported at microgram level using ultra performance liquid chromatography (UPLC)¹², High powered liquid chromatography (HPLC)¹³⁻¹⁴, reversed phase high powered liquid chromatography (RP-HPLC)¹⁵⁻¹⁶, spectrophotometric¹⁷⁻¹⁸, electrometric¹⁹ and capillary zone electrophoresis²⁰.

It is important to develop the good bio analytical method with proper deuterated or analogue based internal standards interms of matrix effect and reproducibility. Moreover, Should not consider the runtime always to minimize the analysis rather than reproducibility and stability for long analytical batches.

The present study describes, for the first time, the development and validation of an isocratic LC-MS/MS with highly efficient, more specific and highly sensitive, simple extraction, good linear method for quantitative determination of VT in

human plasma with the small amount of plasma usage.

MATERIALS AND METHODS:

Chemicals and reagents

Varenicline tartarate reference standard (purity, 99.5%) was purchased from Symed labs, Varenicline tartarate d4 was obtained from Torront research chemicals Canada (**Fig.1**). Tertiary butyl methyl ether (TBME), HPLC grade methanol and acetonitrile were purchased from J.T. Baker USA. Potassium dihydrogen phosphate (KH₂PO₄, reagent grade), Ammonium formate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Human plasma was obtained from Navajeevan blood bank, Hyderabad, India. Ultra pure water from MilliQ-system (Millipore) was used through the study.

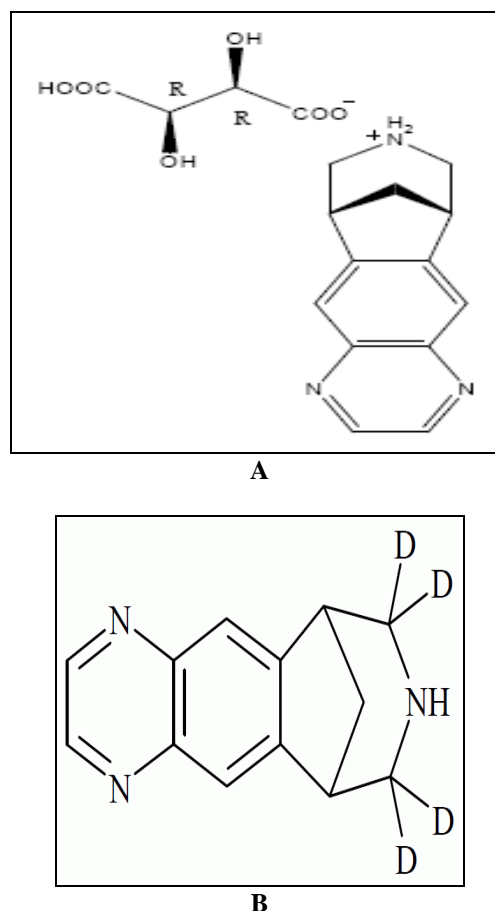


FIG.1: CHEMICAL STRUCTURES OF A) VARENICLINE TARTARATE (VT) B) VARENICLINE TARTARATE D4 (VTd4)

Instrumentation:

The 1200 Series HPLC system (Agilent Technologies, Germany). Mass spectrometric

detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Detection:

The mass transitions were selected as m/z 212.1/169.0 and m/z 215.29/169.1 for quantification of VT and VTd4 respectively. (Fig.2 and 3).

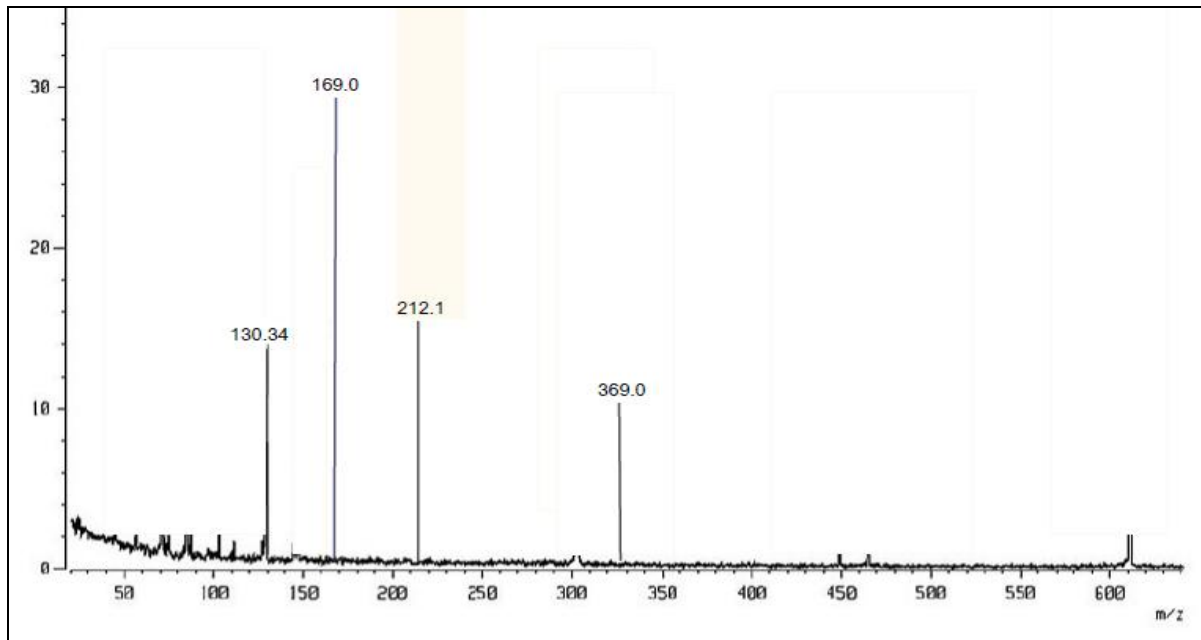


FIG.2: MRM SCAN OF VARENICLINE TARTARATE (VT)

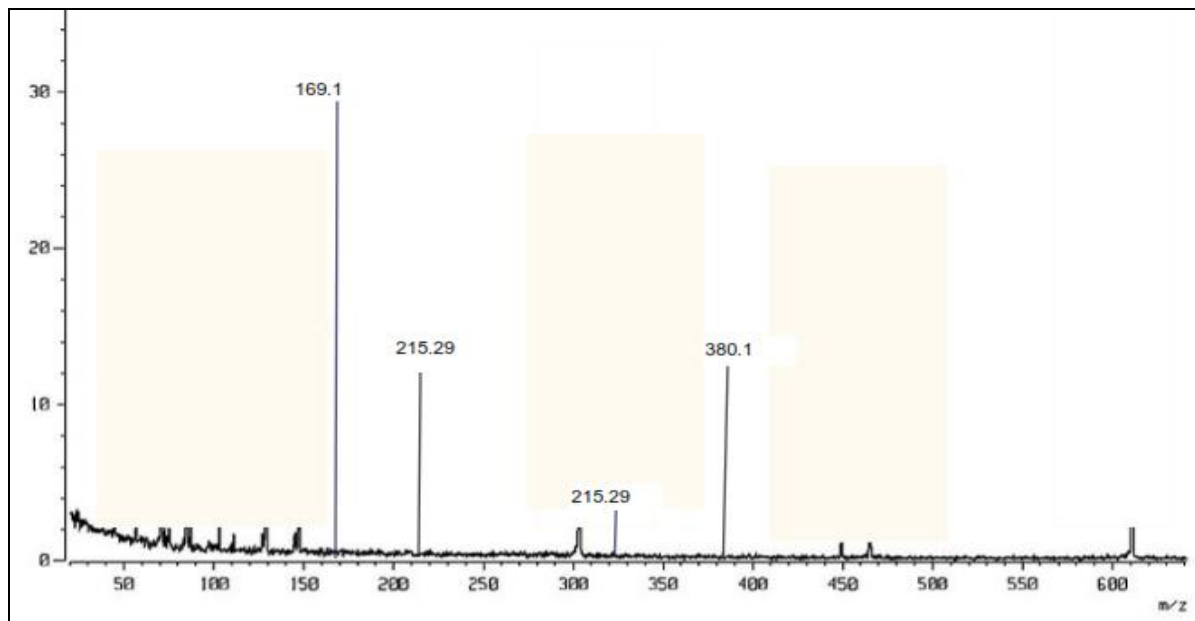


FIG.3: MRM SCAN OF VARENICLINE D4 (VTd4)

Chromatographic conditions:

Zorbax SB-C18, 4.6 x 75 mm, 3.5 μ m 80 \AA analytical column, mobile phase composition of 5 mM ammonium formate: acetonitrile (10:90v/v) with a flow-rate of 0.6 mL/minutes. The column was placed at a temperature of 40°C. 20 μ L of

sample was injected into LC-MS/MS System. The analyte and internal standard were eluted at 9.2 (VT), 8.2 (VTd4) minutes with total runtime of 13 minutes for each injection.

Calibration standards and quality control**Samples:**

Standard Stock solutions of VT (100.0 μ g/mL) were prepared in methanol. From each stock solution 500.0ng/mL, 25.0ng/mL, 2.5ng/mL intermediate dilutions were prepared in plasma. Aliquots of 500.0ng/mL, 25.0ng/mL and 2.5ng/mL were used to spike blank human plasma in order to obtain calibration curve standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0 and 10000.0 pg/mL. Four levels of QC concentrations at 50.0, 150.0, 3000.0 and 8000.0 pg/mL (LLOQ, LQC, MQC and HQC) were prepared by using the different plasma. Spiked calibration curve standards and Quality control standards were stored at -30 °C. Standard stock solutions of VTd4 (100.0 μ g/mL) were prepared in methanol. VTd4 was further diluted to 30.0 ng/mL (Spiked concentration of internal standard) using 50% methanol and stored in the refrigerator 2-8⁰C until analysis.

Sample preparation:

Liquid-liquid extraction was carried out to extract the drug and IS for this purpose 100 μ L of respective concentration of plasma sample was taken into polypropylene tubes and mixed with 50 μ L of internal standard (30.0 ng/mL). This was followed by addition of 100 μ L of 10mM KH₂PO₄ solution and 2.5 mL of methyl tertiary butyl ether and vortexed for approximately 5 minutes. Then the Samples were centrifuged at 4000 rpm for 10 minutes at 20°C. Further, the supernatant was transferred into labeled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then the samples were reconstituted with the reconstitution solution (Acetonitrile: 5mM ammonium formate (90:10)) and vortexed for 2 minutes. Finally, Sample was transferred into auto sampler vials to inject into the LC-MS/MS.

Selectivity and specificity

Selectivity was performed by analyzed the human blank plasma samples from six different sources (donors) with an additional hemolysed group and lipedimic group to test for interference at the retention times of analytes. The peak area of VT in blank samples should not be more than 20% of mean peak area of LOQ of VT. Similarly, peak area of VTd4 in a blank sample should not be more than 5% of mean peak area of LOQ of VTd4.

Precision and accuracy:

Precision and accuracy was determined by replicate analysis of quality control samples ($n = 5$) at LQC (low quality control), MQC (medium quality control) and HQC (high quality control) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

Matrix effect:

The matrix effect due to plasma was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (Liquid-liquid extraction with MTBE) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (% CV) of $\leq 15\%$ was maintained.

Recovery:

The extraction efficiencies of VT and VTd4 were determined by analysis of six replicates at each quality control concentration level and at one concentration for the internal standard VTd4. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non extracted standards (spiked into mobile phase).

Limit of detection and quantification (LOD and LOQ):

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of VT.

Stability (Freeze- thaw, Auto sampler, Room temperature, Long term):

Stock solution stability was performed by comparing the area response of analyte and internal

standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % Change is less than 15% as per US FDA guidelines²¹⁻²³. The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 48 h. The stability of spiked human plasma samples stored at -30°C in autosampler (autosampler stability) was evaluated for 55.5 h.

The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the autosampler at 20°C for 55.5 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 20°C for 27 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long term stability evaluation the concentrations obtained after 71 days were compared with initial concentrations.

RESULTS AND DISCUSSION:

Method development and validation:

The goal of this work is to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of VT from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The MS optimization was performed by direct infusion of solutions of VT and VT d4 into the ESI source of the mass spectrometer. The vital parameters like ionization type, temperature, voltage, gas parameters such as nebulizer and heater gases, compound parameters like DP, EP, FP, CE and CXP were optimized to obtain a better spray shape and ionization to form the respective productions from the protonated VT and VT d4 molecules. Chromatographic conditions especially, composition of the mobile phase, selection of suitable column was optimized through several trials to achieve the best resolution and increase the signal of analyte and internal standard. Different extraction methods like solid phase extraction, Liquid-liquid extraction, precipitation methods were optimized for extraction of VT and VT d4 from the plasma sample.

A good separation and elution were achieved using 5 mM ammonium formate: acetonitrile (10:90v/v) as the mobile phase, at a flow-rate of 0.6 mL/minutes and injection volume of 20 μL . Liquid-liquid extraction was chosen to optimize the drug and internal standard. The retention time was optimized 9.2 minutes for VT and 8.2 minutes for VT d4 (**Fig.4**).

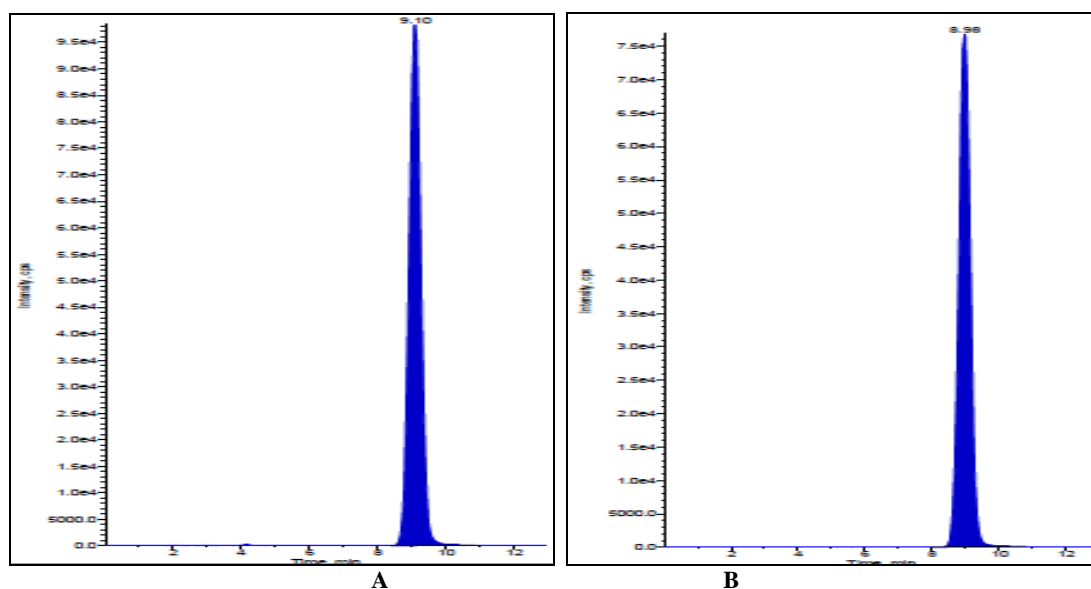


FIG.4: CHROMATOGRAMS OF A) VARENICLINE TARTARATE (VT) B) VARENICLINE TARTARATE D4 (VTd4)

Linearity:

Calibration curve was plotted as the peak area ratio (VT/VT d4) versus (VT) concentration. Calibration was found to be linear over the concentration range

of 50.0 – 10000.0 pg/mL. The correlation coefficient (r^2) was greater than 0.9997 for all curves (**Table 1**).

TABLE 1: CALIBRATION CURVE DETAILS OF VARENICLINE

Spiked plasma concentration (pg/mL)	Concentration measured(mean) (pg/mL) (n = 5)	Precision RSD. ^a (n = 5)	Accuracy %
50.0	51.0 ± 1.3	2.5	102.00
100.0	96.6 ± 4.7	4.9	96.60
500.0	498.4 ± 24.7	5.0	99.68
1000.0	1000.0 ± 17.1	1.7	100.00
2000.0	2013.0 ± 74.6	3.7	100.65
4000.0	4008.4 ± 206.6	5.2	100.21
6000.0	5956.5 ± 190.7	3.2	99.28
8000.0	7952.2 ± 165.6	2.1	99.40
10000.0	10317.1 ± 487.6	4.7	103.17

^a [Standard deviation/mean concentration measured] x 100

Selectivity:

The selectivity of the method was assessed by comparing chromatograms of blank plasma. There were no significant endogenous peaks were observed at respective retention time of VT and VT d4. The results indicate that the method exhibited both good specificity and selectivity.

Between-run variations at three concentrations (150.0, 3000.0 and 8000.0 pg/mL) of QC samples in six replicates. As shown in (**Table 2**) the Within-run Precision and Accuracy were between 1.2 to 4.5 and 91.7 to 105.5 % for VT. Similarly, the Between-run Precision and Accuracy were between 3.5 to 7.4 and 103.9 to 110.6 % for VT. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Precision and Accuracy:

Precision and accuracy for this method were controlled by calculating the Within-run and

TABLE 2: PRECISION AND ACCURACY (ANALYSIS WITH SPIKED PLASMA SAMPLES AT THREE DIFFERENT CONCENTRATIONS)

Spiked plasma concentration (pg/mL)	Within-run (n=6)			Between-run (n=6)		
	Concentration measured (pg/mL) (mean±S.D.)	Precision RSD ^a	Accuracy %	Concentration measured (pg/mL) (mean±S.D.)	Precision RSD ^a	Accuracy %
50.00	51.4±2.3	4.5	102.7	55.5±4.1	7.4	110.6
150.00	154.9±3.4	2.2	105.5	157.9±5.7	3.6	105.2
3000.00	3103.8±102.0	3.3	103.2	3133.0±108.2	3.5	104.3
8000.00	7297.1±89.9	1.2	91.7	7278.7±275.5	3.8	103.9

^a [Standard deviation/mean concentration measured] x 100.

Matrix effect:

The ion suppression/enhancement in the signal at MQC level was found % CV 1.27. These results indicating that there is no effect on ion suppression and ion enhancement.

±2.7 and 97.60 ± 4.7 %. The overall average recoveries of VT and VT d4 were found to be 95.1 ± 6.1 and 98.1 ± 4.47%. Recoveries of the analyte and IS were consistent, precise and reproducible.

Recovery:

The % recoveries of VT were determined at three different concentrations 150.0, 3000.0 and 8000.0pg/mL, were found to be 99.6 ±3.53, 88.2

Limits of Detection and Quantification (LOD&LOQ):

The LOQ& LOD signal-to-noise (S/N) values found for six injections of VT at LOQ & LOD concentrations were 31.95pg/ml and 10.5pg/ml.

Stability (Freeze - thaw, Auto sampler, Room temperature, Long term):

Stock solution stability was performed to check stability of VT and VTd4 in stock solutions prepared in methanol and stored at 2-8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 26 days. The % change for VT and VTd4 were -0.02% and 0.03% respectively indicate that stock solutions were stable at least for 26 days. Room temperature and autosampler stability for VT was investigated at LQC and HQC levels.

The results revealed that VT was stable in plasma for at least 48 h at room temperature, and 78 h in an auto sampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with VT at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that VT was stable in a matrix up to 71 days at a storage temperature of -30°C. The results obtained from all these stability studies are tabulated in (Table 3). Precision (% CV) is less than 5 for Room temperature, long-term, Freeze thaw, auto sampler stability.

TABLE 3: STABILITY OF VARENICLINE IN HUMAN PLASMA SAMPLES

Stability experiments	Storage condition	Spiked plasma concentration (pg/ml)	Concentration measured (n=6) Mean ± SD	RSD ^a (n=6) (%)	Accuracy (%)
Bench top in plasma	RT	150.0	148.3 ± 8.1	5.5	98.9
	48 hr	8000.0	6728.3±206.3	3.1	81.5
Processed (extracted sample)	Autosampler	150.0	162.3 ± 2.4	1.5	108.2
	55.5 hr	8000.0	7536.7±294.5	3.9	90.4
Freeze/Thaw stability	-30°C	150.0	156.5 ± 4.0	2.5	104.3
	Cycle-3	8000.0	7381.7±173.4	2.3	90.4
Long-term stability in human plasma	-30°C	50.0	160.3±13.2	8.2	106.9
	71 days	8000.0	7450.0±229.1	3.1	90.5

^a [Standard deviation/mean concentration measured] x 100

CONCLUSION: The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other described methods in previously. Quantification of Varenicline tartrate was compared with respective isotope labeled internal standards. Extraction of analyte and IS were achieved by using LLE. Linearity range, column, mobile phase, flow rate, injection volume, plasma usage volume for analysis was improved. Hence this method has significant advantages over previously reported methods in-terms of Selectivity, sensitivity, Linearity, Reproducibility.

ACKNOWLEDGEMENTS: Authors wish to thank the support received from ICT (Indian institute of chemical technology) Hyderabad India for providing Literature survey, APL Research Pvt. Ltd. Hyderabad India to carry out this Research work.

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How to cite this article:

Dasari PK and Darapaneedi SR: Picogram Level Quantification of Varenicline in Human Plasma Samples BY LC-ESI-MS/MS. *Int J Pharm Sci Res* 2016; 7(6): 2510-17. doi: 10.13040/IJPSR.0975-8232.7(6).2510-17.

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